

AAV9 Delivery of shRNA to the Mouse Heart

UNIT 23.16

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RNA interference (RNAi) is a rapid approach to dissect loss-of-function phenotype for a gene of interest. However, it is challenging to perform RNAi in specific organs and tissues *in vivo*. Engineered viruses can provide a useful tool for delivery of small RNAs *in vivo*. Recombinant adeno-associated viruses (rAAVs) are the preferred method for delivering genes or gene modulators to target cells due to their high titer, low immune response, ability to transduce many types of cell, and overall safety. In this unit, we describe protocols for use of rAAVs as a cargo to deliver miRNA backbone-based shRNA controlled by a cardiac-specific promoter into the mouse heart. © 2016 by John Wiley & Sons, Inc.

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INTRODUCTION

RNA interference (RNAi) has become a routine and robust tool to study loss-of-function phenotype in a gene of interest. Gene silencing by RNAi can be achieved through direct transfection of a 21 base pair double-stranded RNA duplex or plasmid expressing a short hairpin RNA (shRNA) cassette into culture cells (Zamore, 2001). Genome-wide RNAi screening has been successfully applied to identify key regulators in stem cells, cancer cells, and even in lower model organisms such as *Caenorhabditis elegans* and *Drosophila* (Kamath et al., 2003; Dietzl et al., 2007; Luo et al., 2009). Therefore, it is of great interest to apply RNAi to widely used mouse models. Successful reports include combination of transgenic or gene-targeting technology with RNAi. However, to achieve this end, an animal facility with special equipment and well-trained personnel is required (Kunath et al., 2003; Coumoul and Deng, 2006). With viral tools, especially recombinant adeno-associated viruses (rAAVs), shRNA or transgenes can be delivered into specific organs and tissues *in vivo*. Newly discovered serotype AAVs, such as AAV8, AAV9, and AAVrh10, are able to cross the endothelium and deliver the transgene or shRNA throughout the body (Jiang et al., 2013; Lin et al., 2014; Xiong et al., 2015). We and collaborators have used AAV9 coupled with the cardiac-specific promoter cTnT to perform RNAi and deliver transgenes, shRNA, and Cre specifically into cardiac cells (Jiang et al., 2013; Lin et al., 2014; Ding et al., 2015; Lin et al., 2015; Prendiville et al., 2015; Xiong et al., 2015). In this unit we describe protocols to determine the RNAi efficiency and construct the AAV-shRNA (Basic Protocol 1), to produce and purify the AAV-shRNA (Basic Protocols 2 and 3), and to deliver the AAV-shRNA into a mouse heart (Basic Protocol 4).

Manipulating the Mouse Genome

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CAUTION: All virus work should be performed in a dedicated tissue culture hood and incubator separate from those used for maintaining cell lines. Proper disposal of virally contaminated materials is essential.

DETERMINATION OF RNAi EFFICIENCY AND CONSTRUCTION OF AAV-shRNA

Cardiac-specific genes are silenced in commonly used cell lines. To assess RNAi efficiency, we developed a two-color system (GFP and Cherry) to measure the knockdown effect of shRNA constructs in vitro. This protocol outlines the steps necessary to select the best shRNA constructs.

Materials

pCAGIG plasmid (Addgene plasmid no. 11159)
LB agarose plates containing 100 µg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
AAV vectors (available from Dr. Jiang Jianming):
 pCAG-cherry-miR155
 pAAV-EGFP-miR155
Competent cells:
 Top10 (Thermo Fisher Scientific, cat. no. C4040-10)
 Stbl3 (Thermo Fisher Scientific, cat. no. C7373-03)
LB medium containing 100 µg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
QIAGEN Plasmid Miniprep Kit
Cloning primers for gene of interest
Restriction enzymes appropriate for cloning primers
BsmBI restriction enzyme (New England Biolabs)
shRNA primers (BLOCK-iT miR RNAi; Thermo Fisher Scientific)
HEK293T cells (ATCC)
Complete DMEM (see recipe)

Sterile pipet tips
30°C and 37°C shaking incubator
Nanodrop spectrophotometer
6-well culture plate
Fluorescence microscope

Additional reagents and equipment for PCR (*UNIT 15.1*; Kramer and Coen, 2001), DNA digestion (*UNIT 3.1*; Bloch, 2001), DNA ligation (*UNIT 15.3*; Mueller et al., 2002), RNA extraction (*UNIT 4.1*; Gilman, 2002), and real-time PCR (*UNIT 15.8*; Bookout et al., 2006)

Prepare pCAGIG, pCAG-cherry-miR155, and pAAV-EGFP-miR155 plasmids

1. Touch a sterile pipet tip to the bacterial stock (pCAGIG) from the bacterial stab, and streak it onto an LB/ampicillin plate. Incubate plate at 37°C overnight.
2. Transform the pCAG-cherry-miR155 and pAAV-EGFP-miR155 into Top10 and Stbl3 competent cells, respectively, following the instructions provided by the manufacturer. Incubate Stbl3 plate at 30°C overnight.

AAV vectors are available from authors upon request. Contact Dr. Jiang Jianming at bchjian@nus.edu.sg.

Since AAV vector pAAV-EGFP-miR155 contains two ITRs, we prefer to grow bacteria with this AAV vector at 30°C to reduce vector recombination.

3. Once colonies are formed, pick a single colony from the plate to inoculate 5 ml LB liquid medium containing 100 µg/ml ampicillin. Grow pCAGIG or pCAG-cherry-miR155 clones overnight with shaking at 37°C, 200 rpm. Grow pAAV-EGFP-miR155 clones overnight with shaking at 30°C, 200 rpm.

pAAV-EGFP-miR155 will be used to clone and make the AAV.

Since AAV vector pAAV-EGFP-miR155 contains two ITRs, we prefer to grow bacteria with this AAV vector at 30°C to reduce vector recombination.

4. Isolate plasmids using a Miniprep Kit. Measure the DNA concentration (e.g., using a Nanodrop).

Clone gene of interest into pCAGIG

5. Design the cloning primers for the gene of interest with suitable restrictive enzymes sites.

Multiple cloning sites of pCAGIG include EcoRI, XhoI, EcoRV, and NotI.

6. Perform PCR for the gene of interest, digest PCR product with suitable restriction enzymes, and ligate the digested PCR product into the pCAGIG vector.
7. Transform the ligation product into Top10 competent cells.
8. Isolate plasmids by Miniprep Kit, and submit plasmid for Sanger sequencing.

Clone RNAi sequence into pCAG-cherry-miR155

9. Digest pCAG-cherry-miR155 with BsmBI.
10. Anneal shRNA primers and ligate annealed primers into pCAG-cherry-miR155.

Order pre-designed BLOCK-iT miR RNAi (Thermo Fisher Scientific), and select hairpin primers (<https://rnaidesigner.thermofisher.com/rnaiexpress/rnaiExpress.jsp>). Primers are designed and selected using a unique set of parameters to reduce the potential of off-target effects, such as mismatch alignment. After primer annealing, overhang of the hairpin is TGCT at the 5' end and CCTG at the 3' end, which can be inserted into the pCAG-cherry-miR155 vector digested with BsmBI.

11. Transform the ligation product into Stbl3 competent cells.
12. Isolate plasmids by Miniprep Kit, and submit plasmid for Sanger sequencing.

Transfect human HEK293T cells

13. Culture HEK293T cells in complete DMEM in 6-well plates until the cells are ~80% confluent.
14. Cotransfect pCAGIG (gene of interest) and pCAG-cherry-miR155 (shRNA) into HEK293T cells.
15. Observe the red and green signal under a fluorescence microscope.

Red fluorescence signal indicates transfection efficiency. You will observe similar red fluorescence in all transfected cells.

GFP is coexpressed with the gene of interest. The GFP signal is drastically reduced if RNAi works.

16. (Optional) Extract total RNA, reverse transcribe, and test knockdown efficacy by real-time PCR.

Clone RNAi sequence into pAAV-EGFP-miR155 to make AAV-shRNA

17. Digest pAAV-EGFP-miR155 with BsmBI.

18. Anneal the DNA sequences encoding the selected shRNA primers, and ligate the annealed primer DNA into pAAV-EGFP-miR155 digested with BsmBI.
19. Transform the ligation product into Stbl3 competent cells.
20. Isolate plasmids by Miniprep Kit, and submit plasmid for Sanger sequencing.

PRODUCTION OF AAVs

The AAV is generated by transient transfection of HEK293T cells using three plasmids: (1) AAV-shRNA, the cis-ITR-containing plasmid; (2) pAAV2/9, the trans-plasmid encoding AAV replicase and capsid gene; and (3) pAdDeltaF6, the adenoviral helper plasmid. This protocol outlines the steps necessary to produce the AAV in HEK293T cells.

Materials

pAAV2/9 (available from University of Pennsylvania Penn Vector Core)
pAdDeltaF6 (available from University of Pennsylvania Penn Vector Core)
QIAGEN Plasmid Maxi Kit
HEK293T cells (ATCC)
Transfection reagent (polyethylenimine [PEI]; e.g., Polysciences)
pAAV-EGFP-miR155 (available from Dr. Jiang Jianming)
Complete DMEM (see recipe)

15-cm culture plates
Centrifuge

1. Purify pAAV2/9 and pAdDeltaF6 plasmids using a QIAGEN Plasmid Maxi Kit.
2. Seed HEK293T cells into 10 culture plates 20 to 24 hr before transfection.
3. Cotransfect the 3 plasmids until ~80% confluence in 10 culture plates of HEK293T cells from step 2 using PEI (Godbey et al., 1999):

70 μ g pAAV2/9
70 μ g pAAV-EGFP-miR155
200 μ g pAdDeltaF6

pAAV-EGFP-miR155 is available from authors upon request. Contact Dr. Jiang Jianming at bchjian@nus.edu.sg.

The HEK293T cell line is relatively easy to transfect. Since we transfect 10 culture plates, a cost-effective transfection reagent (i.e., PEI) is recommended to reduce the cost of AAV production.

4. Change medium 24 hr after transfection with complete DMEM.
5. After transfection (60 hr), dislodge cells by pipetting up and down with the culture medium.
6. Collect all cells from each prep (10 plates total) by centrifuging 10 min at 2000 rpm ($750 \times g$), 20°C, and remove supernatant.

The yield of cells should be around 5 ml.

The pellet can be stored at -80°C for 1 to 2 months.

PURIFICATION OF AAV BY IODIXANOL GRADIENT ULTRACENTRIFUGATION

Iodixanol gradient ultracentrifugation is a rapid and reproducible method to purify recombinant AAV. This protocol outlines the steps necessary to purify AAVs produced in HEK293T cells.

Materials

HEK293T cells containing AAV (see Basic Protocol 2)
Lysis buffer (see recipe)
Dry ice/ethanol bath
Magnesium chloride
Benzonase Nuclease (e.g., Sigma)
17%, 25%, 40%, and 60% (w/v) iodixanol (see recipe)
PBS (*APPENDIX 2*)

Vortex
37°C water bath
Dounce tissue grinders (e.g., Wheaton)
Centrifuge (e.g., Beckman)
Beckman OptiSeal Polypropylene Tube, 36.2-ml, 25 mm × 87 mm
20-cm blunt-end needle
Ultracentrifuge (e.g., Beckman)
Ultracentrifuge rotor (e.g., Beckman VTi 50)
19-G needles
3-ml and 10-ml syringes
Amicon Ultra-15 Centrifugal Filter Units (nominal molecular weight limit of 100 kD)
50-ml Falcon tubes
1.5-ml microcentrifuge tube

Perform iodixanol gradient centrifugation

1. Resuspend the HEK293T cell pellet in 15 ml lysis buffer by gently vortexing.
2. Freeze-thaw the cell pellet three times using a dry ice/ethanol bath and 37°C water bath.

Make sure the cells freeze well in the dry ice/ethanol bath and then thaw completely in the water bath.
3. Add MgCl₂ to a final concentration of 1 mM.
4. Add Benzonase to a final concentration of 250 U/ml. Mix by gently vortexing, and incubate at 37°C for 15 min.
5. Homogenize the cell lysates 20 times using Dounce tissue grinders at room temperature. Gently push and pull the glass pestle to avoid bubble formation.
6. Centrifuge the cell debris 30 min at 3000 rpm (5000 × g), 4°C, twice.
7. Prepare iodixanol gradient.
 - a. In the OptiSeal tube, add 6 ml of 17% iodixanol using a 20-cm blunt-end needle.
 - b. Add 6 ml of 25% iodixanol using a 20-cm blunt-end needle.
 - c. Add 5 ml of 40% iodixanol using a 20-cm blunt-end needle.
 - d. Fill up the remainder of the tube with 6 ml of 60% iodixanol using a 20-cm blunt-end needle.

8. Transfer lysate on top of the gradient.
9. Ultracentrifuge 90 min at 48,000 rpm ($225,000 \times g$), 16°C.

Harvest and purify AAV

10. Harvest viral fraction: Use a 19-G needle and insert the needle 2 mm below the intersection of the 60% and 40% layers. Collect 3 ml of the 40% layer but avoid the 25% layer.

The 60% layer will be yellow in color, and the 40% layer will be transparent. The virus will be in the 40% fraction.

11. Add PBS to viral fraction to bring total volume to 15 ml. Mix well using a pipet, and transfer solution into Amicon Ultra-15 Centrifugal Filter Units.

This step is used to remove the iodixanol and concentrate the virus.

12. Centrifuge 30 min at 3500 rpm ($5000 \times g$), 4°C. Change column to a new 50-ml Falcon tube to remove the bottom fraction, fill top again with PBS (up to 15 ml), and mix well by pipetting up and down to ensure that the remainder of the iodixanol is well mixed for optimal removal. Repeat centrifugation and PBS; wash two additional times.

After the last centrifugation, you will observe 200 to 250 μ l of clear solution on the bottom of the column which contains the concentrated AAV virus.

13. Transfer the clear solution containing the virus to a 1.5-ml microcentrifuge tube, and store at -80°C .

BASIC PROTOCOL 4

AAV TRANSDUCTION IN VIVO

After purification of AAV, the next step is to transduce the virus into the mouse heart. This protocol outlines the steps necessary to inject AAVs into the thoracic cavity of neonatal mouse pups.

Materials

- Mouse with newborn pups
- Purified AAV (see Basic Protocol 3)
- 3M Micropore Medical Tape
- BD insulin syringe equipped with BD Ultra-Fine Needle, 31-G \times 5/16" (0.3-ml \times 8 mm)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

1. Perform cryoanesthesia according to institutional guidelines, and place the pups in dorsal recumbency using 3M Micropore tape.
2. Prepare an insulin syringe and load 50 μ l AAV.
3. Place the tip of the needle at the level of the xiphoid process and \sim 2 mm to the left of the xiphoid process.
4. Puncture the skin and diaphragm while directing the needle to avoid the heart and lung at an angle of \sim 40° to 45°.
5. Slowly inject 50 μ l AAV into the thoracic cavity.
6. Remove the syringe and dispose of according to institutional regulations.

7. Return the pups to the adult mouse.

The injection is a minimally invasive procedure, and pups will be immediately recognized by the adult mouse.

REAGENTS AND SOLUTIONS

Use molecular biology grade (nucleic acid- and nuclease-free) or sterile-filtered double-deionized water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2.

Complete DMEM

High-glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen)
supplemented with:
10% fetal bovine serum (FBS)
50 μ g/ml gentamicin (Invitrogen)
Filter sterilize
Store up to 3 months at 4°C

Iodixanol gradient solutions

17%:

5 ml 10 \times PBS (APPENDIX 2)
0.05 ml 1 M MgCl₂
0.125 ml 1 M KCl
10 ml 5 M NaCl
12.5 ml OptiPrep (iodixanol)
H₂O to 50 ml

25%:

5 ml 10 \times PBS (APPENDIX 2)
0.05 ml 1 M MgCl₂
0.125 ml 1 M KCl
20 ml OptiPrep (iodixanol)
0.1 ml 0.5% phenol red
H₂O to 50 ml

40%:

5 ml 10 \times PBS (APPENDIX 2)
0.05 ml 1 M MgCl₂
0.125 ml 1 M KCl
33.3 ml OptiPrep (iodixanol)
H₂O to 50 ml

60%:

0.05 ml 1 M MgCl₂
0.125 ml 1 M KCl
50 ml OptiPrep (iodixanol)
0.025 ml 0.5% phenol red

Lysis buffer

150 mM NaCl
20 mM tris·Cl, pH 8.0
Filter sterilize
Store up to 6 months at 4°C

COMMENTARY

Background Information

Rapid and safe *in vivo* gene transfer technology is very important for studies in animal models and clinical applications. AAVs are able to transfer genes efficiently to different tissues and enable long-term gene modulation. In addition, AAVs can be administered to the heart without surgical procedures. Besides having low immunogenicity, AAVs have not been shown to be associated with any known symptoms or pathology in humans to date (Vandenberghe et al., 2009). More importantly, the use of AAVs as a gene therapy vector has been approved recently for clinical application (Bryant et al., 2013).

AAVs can be used as a cargo to deliver transgenes and shRNA into the heart. miRNA backbone-based shRNA co-cistronically expressed with a fluorescent marker gene can be driven by a pol II tissue-specific promoter. The strong correlation of fluorescent marker expression with the knockdown activity helps to track the presence of shRNA in fluorescent-positive cells.

Critical Parameters

Intact vectors

For amplification of the AAV expression vector, we use Stbl3 competent cells which will minimize recombination that can occur through the two ITRs and result in a loss of part of the vector.

Size limit

A primary limitation of the AAV is its limited effective packaging capacity of ~4.7 kb. After removing the cardiac promoter and polyA signal, the actual packaging capacity is ~3.2 kb. The size of miRNA backbone-based shRNA, together with EGFP, is <1.2 kb which is within the packaging capacity of the AAV.

Troubleshooting

Low transfection efficiency for HEK293T cells

Suggestion: Prepare highly purified DNA, and make sure that cells are healthy and within low passage.

No knockdown of gene of interest

Suggestions: Test shRNA construct in cell lines expressing the gene of interest; or derive myocytes from heart

Anticipated Results

We often achieve ~60% to 90% transduction efficiency using the protocol described in this unit. Success and efficacy of RNAi depends on the RNAi targeting region and stability of targeted transcript and protein.

Time Considerations

Basic Protocol 1

Cloning overexpression (pCAGIG) and preparation of shRNA (pCAG-Cherry-miR155) constructs takes 2 to 3 days. Measuring RNAi efficacy takes 2 to 3 days.

Basic Protocols 2 and 3

Production and purification of AAVs takes 5 to 6 days.

Basic Protocol 4

Virus transduction takes 2 to 3 hr. After 1 to 2 weeks, we can assess expression of the transgene and RNAi in the mouse heart.

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